Conjugates of Poly(*N*-isopropyl acrylamide-*co*-acrylic acid) with Alanine Monopeptide, Dipeptide, and Tripeptide

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ABSTRACT: A random copolymer of *N*-isopropyl acrylamide (NIPAAm) and acrylic acid (AAc) with an AAc content of 3.1 ± 0.19 mmol of carboxylic acid groups per gram of the copolymer and with a number-average molecular weight of 1400 was synthesized by free-radical polymerization with 2,2′-azoisobutyronitrile in dimethylformamide. Then, monopeptide, dipeptide, and tripeptide (i.e., alanine) conjugates of this copolymer were prepared with their carboxyl-end-protected (with methyl ester hydrochloride) form of alanine, with a water-soluble carbodiimide. Of the carboxylic acids, 93, 69, and 57% were conjugated (loaded) with alanine at the monopeptide, dipeptide, and tripeptide conjugation steps, respectively. The chemical structures of the copolymer and conjugates were analyzed by Fourier transform infrared and ^{1}H-NMR, which revealed the conjugate formation. Amino acid conjugation caused significant decreases in the lower critical solution temperatures (LCST) of the copolymer, especially at pH 7.4. The LCST values of the dipeptide and tripeptide conjugates of poly(NIPAAm-*co*-AAc) at both pH 4.0 and 7.4 shifted to significantly higher temperatures. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 2012–2019, 2003

Key words: stimuli-responsive; oligopeptides; prodrugs; LCST

INTRODUCTION

Peptides, glycopeptides, oligopeptides, and their conjugates with carrier molecules have great potential in therapeutic and protective medicine as drugs (or prodrugs) and vaccines.[1–3] Soluble polymers, especially poly(ethylene glycol)s, have attracted attention as carriers of peptides.[4,5] The soluble character of these polymers allows one to carry out the desired manipulations on the conjugates in a homogeneous solution state by preserving those macromolecular properties that meet the particular requirements for an application. Numerous poly(ethylene glycol)/peptide conjugates show improved physicochemical and pharmacological properties, such as extended plasma half-life, reduced antigenicity, increased solubility, resistance to proteolysis, and higher biological activity.[6–9]

Stimulus-responsive polymers exhibit large reversible physical changes in response to external stimuli such as the temperature, pH, ionic strength, solvents, and radiation.[10–12] The most popular of these types of polymers is poly(*N*-isopropyl acrylamide) (PNIPAAm), which exhibits a temperature-sensitive character: the polymer chains change from water-soluble coils into water-insoluble globules in an aqueous solution as the temperature increases above the lower critical solution temperature (LCST).[13] The copolymerization of *N*-isopropyl acrylamide (NIPAAm) with acrylic acid (AAc) allows the synthesis of both pH- and temperature-responsive copolymers.[14–16]

Recently, we attempted to prepare water-soluble peptide (or oligopeptide)–polymer conjugates that would allow us to design prodrug formulations with novel stimulus-responsive characteristics, which may increase the stability and effectiveness and allow targeted delivery (especially into the cells) of the bioactive peptides or oligopeptides. The methods of synthesizing NIPAAm and AAc copolymers and their stimulus-responsive properties have been reported previously.[14,17] Here we present the procedure for the synthesis of conjugates of monopeptides, dipeptides, and tripeptides (alanine) and poly(NIPAAm-*co*-AAc).

EXPERIMENTAL

Materials

NIPAAm (Aldrich, United States) was purified by recrystallization from *n*-hexane. AAc (Fluka, Buchs, Switzerland) was redistilled *in vacuo*. 2,2′-Azoisobutyronitrile (AIBN; Fluka) was recrystallized from methanol. The methyl ester hydrochloride of D,L-alanine (Ala-OMe) and the activation agent, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC), were purchased from Sigma (USA). Anhydrous *N*,*N*-dimethylformamide (DMF; Sigma), triethyl amine (TEA; Sigma), and diethyl ether (Merck,

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Haar, Germany) were used without further purification. For ninhydrin tests, ninhydrin, hydrindantin, and dimethyl sulfoxide (DMSO) were obtained from Fluka Chemicals, Merck, and BDH Chemicals, Ltd. (Poole, UK), respectively. All other reagents used were analytical-grade.

Synthesis of the copolymers

NIPAAm and AAc copolymers were synthesized in DMF by a free-radical copolymerization with AIBN as the initiator, which was described in detail elsewhere.[14,17] Briefly, 50 mmol of NIPAAm, 18.2 mmol of AAc, and 2.4 mmol of AIBN were dissolved in 23 mL of DMF in a Pyrex reactor, which was kept in an ice bath. Nitrogen was purged through the solution for 10 min before copolymerization. The reactor was placed in a constant-temperature water bath at 60°C, and the polymerization was continued for 90 min. The copolymer was precipitated with diethyl ether, filtered through a sintered glass filter, washed repeatedly with diethyl ether, and dried *in vacuo* at 40°C for 48 h.

The chemical structure and AAc content of the copolymer were obtained with Fourier transform infrared (FTIR; FTIR 8000, Shimadzu, Japan) and NMR (Bruker, AC250, Billerica, MA) spectrometers. The discs were prepared for the FTIR analysis by the mixing of the dry copolymer samples with KBr crystals (10 wt %). FTIR spectra were taken at room temperature. ^{1}H-NMR spectra of the copolymers were measured at 400 MHz with DMSO-d_6 as a solvent.

The number-average molecular weight of the polymer was determined with both an Ubbelohde automatic viscometer (Schott Gerate, Berlin, Germany) and a vapor pressure osmometer (Knauer, Mainz, Germany) with methanol as the solvent. The viscosity measurements were performed at a constant temperature of 25°C.

LCSTs at pH 4.0 and 7.4 were obtained spectrophotometrically (UV 1602 spectrophotometer, Shimadzu) at 500 nm. A copolymer solution containing 2.0 mg of polymer/mL in acetic acid/acetate (pH 4.0) or phosphoric acid/phosphate (pH 7.4) buffers was used for the LCST measurements. The ionic strength of the buffer solutions was adjusted by the addition of an appropriate amount of NaCl. The temperature at 10% of the maximum absorbance of the copolymer solution was defined as the LCST.

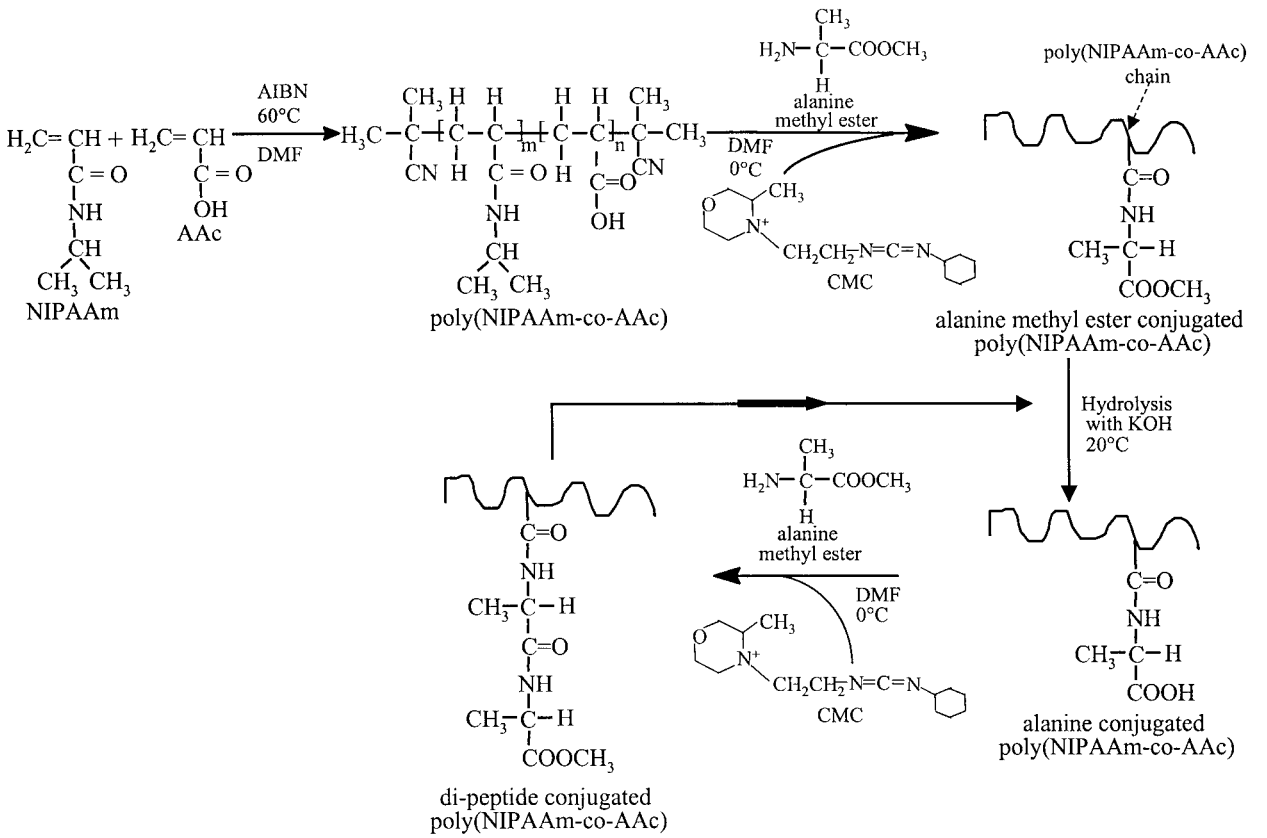
Synthesis of the amino acid/copolymer conjugates

The monopeptide, dipeptide, and tripeptide conjugates were synthesized by using carboxylic-end groups-protected alanine (Ala-OMe) by a three-step procedure. In the first step, a proper amount of Ala-OMe was dissolved in 10 mL of anhydrous DMF in a reactor (a two-necked glass flask) that was kept in a constant-temperature bath at 0 ± 2°C. The amount of Ala-OMe was 3.72 mmol, which was in excess (20 mol %) of the carboxylic acid groups of the copolymer used. The Ala-OMe solution was then neutralized by the addition of an equimolar amount of TEA. One gram of the copolymer and an equimolar amount of CMC were dissolved in 10 mL of anhydrous DMF in a separate flask and then added to the Ala-OMe solution to start the condensation reaction, which was conducted at 0 ± 2°C by continuous stirring with a magnetic stirrer (at 400 rpm) for 15 h.

For the purification of the amino acid/copolymer conjugate, after the reaction, the solvent was removed in a rotary evaporator *in vacuo* at 38°C. The solid obtained was then dissolved in distilled water. The pH of the solution was adjusted to 3.5 by the addition of 3*M* HCl. The polymer was precipitated in a water bath at 40°C in 10 s and separated from the supernatant, which contained the unconverted amino acids, and other wastes by centrifugation (force = 24,000*g*) at 38°C for 2 min with a centrifuge (Biofuge, Herhaus, Germany). After centrifugation, the precipitate was dissolved in distilled water, and the thermal purification procedure was repeated twice. The recovery yields of the copolymer conjugates were determined to be 80 ± 12 wt % by a gravimetric analysis of the copolymer precipitates obtained at the end of each three-step purification process realized after the conjugation and saponification reactions.

For the removal of the protecting group of the amino acid (i.e., methyl ester group), the conjugate was treated in a 10% aqueous KOH solution at a constant temperature of 20°C by continuous stirring with a magnetic stirrer (at 400 rpm) for 20 h. Then, this conjugate was purified with the procedure given in the previous paragraph. The hydrolysis of ester groups to acid groups was monitored by ^{1}H-NMR. The yields obtained in the hydrolysis experiments were 81 and 85 mol % for copolymer/Ala-OMe and copolymer/Ala–Ala-OMe conjugates, respectively. In other words, 81–85 mol % of the ester groups were hydrolyzed to the carboxylic acid groups under the experimental conditions. Here it is worth noting that no hydrolysis with amide bonds on the NIPAAm component of the polymer was observed after the hydrolysis process was used to remove the methyl ester groups of the amino acids, and this was verified by both the ^{1}H-NMR and FTIR data (not shown here).

By the reaction of Ala-OMe with alanine- and dialanine-conjugated copolymers, dipeptide and tripeptide conjugates, respectively, were prepared with exactly the same procedure previously described, except for the temperature applied to the conjugate solutions in the purification process. The precipitation temperature for dipeptide and tripeptide conjugates was determined by the temperature being increased in 5°C



Scheme 1 Synthesis of the poly(NIPAAm-*co*-AAc) copolymer and poly(NIPAAm-*co*-AAc)/alanine conjugates.

increments from 40°C until precipitation was observed.

The amount of amino acid connected to the copolymer was determined by ninhydrin testing.[18] The supernatant solution (150 μL) collected during the washing steps previously described was diluted with distilled water to 3.5 mL. This solution was first incubated with 1.5 mL of the ninhydrin solution, at 100°C for 15 min, and then brought to room temperature by immediate cooling (in an ice bath), and the absorbance was measured with a UV spectrophotometer (UV 1602 spectrophotometer, Shimadzu) at 570 nm. The ninhydrin solution was freshly prepared by the mixing of 25 vol % of the lithium acetate buffer (4*M*, pH 5.2) and 75 vol % of a DMSO solution containing ninhydrin and hydrindantin dihydrate. The concentrations of ninhydrin and hydrindantin in the ninhydrin solution were 2% (w/v) and 3 mg/mL, respectively. The amount of bound amino acid was then calculated from the total amount of amino acid used and the amount left in the supernatant (obtained from the ninhydrin test).

The chemical structures of the conjugates were analyzed by FTIR (FTIR 8000, Shimadzu) and NMR (AC250, Bruker) spectrometers. The LCSTs of the conjugates at pH 4.0 and 7.4 were obtained spectrophotometrically (UV 1602 spectrophotometer, Shimadzu) at 500 nm with the same procedure used for the copolymer described earlier.

TABLE I
Percentage Alanine Conjugated to Copolymer and Amount of Alanine Connected to the Unit Mass of Copolymer

Conjugate	Alanine reacted (%)[a]	Amount of alanine in the conjugate (mmol of alanine/g of copolymer)[b]
Ala-OMe Conjugate	93	2.89
Ala–Ala-OMe Conjugate	69	1.60
Ala–Ala–Ala-OMe Conjugate	57	0.77

[a] Based on the carboxylic acid groups available on the copolymer, calculated from residual amino acids.

[b] Amount of carboxylic acid groups available on the copolymer is 3.1 ± 0.19 mmol/g copolymer.

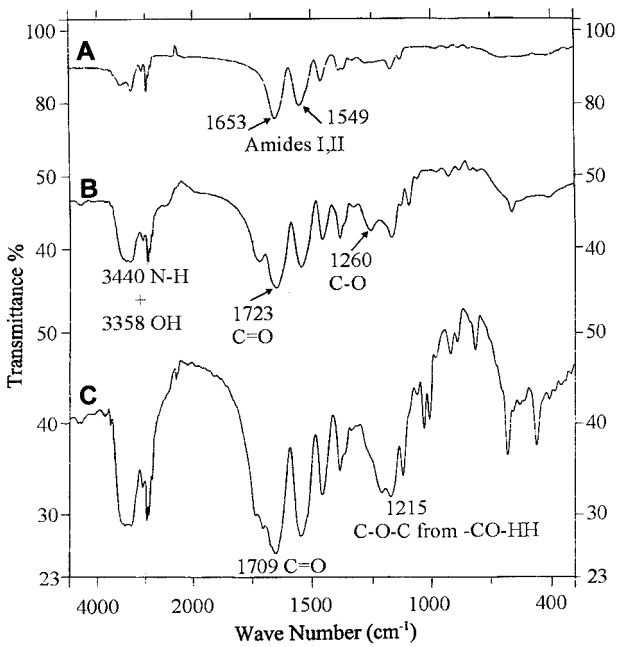


Figure 1 FTIR spectra of (A) PNIPAAm, (B) poly-(NIPAAm-*co*-AAc), and (C) poly(NIPAAm-*co*-AAc)/Ala-OMe.

RESULTS AND DISCUSSION

Conjugation of alanine with poly(NIPAAm-*co*-AAc)

In this study, we attempted to synthesize monopeptide, dipeptide, and tripeptide conjugates of alanine with a temperature- and pH-responsive poly-(NIPAAm-*co*-AAc) copolymer. As schematically shown in Scheme 1, we first synthesized the copolymer from its respective monomers, NIPAAm and AAc, by a free-radical copolymerization with AIBN as the initiator in DMF.[17]

The AAc content of the copolymer synthesized in this study was determined from the ^{1}H-NMR data to be 31.6 mol % (based on total monomers), which corresponded to a carboxylic acid content of 3.1 mmol of COOH per gram of the copolymer.[17] The number-average molecular weight of this copolymer was found to be 1400 g/mol from the data obtained with a viscometer.[17]

Then, we connected Ala-OMe to the copolymer through the amino end with a water-soluble carbodiimide (CMC).[18] This conjugate was purified, hydrolyzed (for the conversion of the methyl ester groups into carboxylic acid groups), and then purified again. The same procedure was employed to synthesize dipeptide and tripeptide conjugates of the copolymer.

The percentage of alanine consumed in the reaction with the copolymer and the amount of alanine connected to the unit mass of the copolymer were determined from the data obtained in the ninhydrin tests previously described. As seen in Table I, the yields of alanine conjugation in all three steps were between 57 and 93% and decreased as the amino acid sequence increased. Lower connection yields were also reported by others in the synthesis of homologous oligopeptides as the length of the oligopeptide chain was increased.[5,19,20] The amounts of alanine connected to the unit mass of the copolymer in alanine, dialanine, and trialanine reactions were 2.89, 1.60, and 0.77 mmol of alanine per gram of copolymer, respectively, much larger than the different polymer values reported in similar studies carried out with different polymeric carriers. For instance, the loading of glycine tetrapeptide to carboxymethyl/poly(ethylene glycol) in solution was only 0.1 mmol of glycine/g of polymer catalyst by an immobilized enzyme.[21] Bayer[22] was able to connect 0.08–0.49 mmol of glycine per gram of poly-(ethylene glycol)s with different molecular weights. In another study, the loading capacities for alanine and valine were 0.2 mmol per gram of poly(ethylene glycol).[20]

Structural analysis

Figure 1(A–C) gives the FTIR spectra of the PNIPAAm homopolymer, poly(NIPAAm-*co*-AAc) copolymer,

TABLE II
Characteristic Peaks in the FTIR Spectra of PNIPAAm, Poly(NIPAAm-*co*-AAc) and Poly(NIPAAm-*co*-AAc) Ala-OMe

Characteristic peak		Wave number (cm^{-1})
—$CH(CH_3)_2$	C—H bending	1389
—CH	C—H stretching	2976
—CH_2	C—H stretching	2986
—CONH	Amide I (C═O stretching)	1650
	Amide II (N—H bending)	1547
	N—H stretching	3400
—COOH	C═O stretching	1723
	O—H stretching	3357
	C—O stretching	1260
—$COOCH_3$	C═O stretching	1709
	C—O—C asymmetric stretching	1215

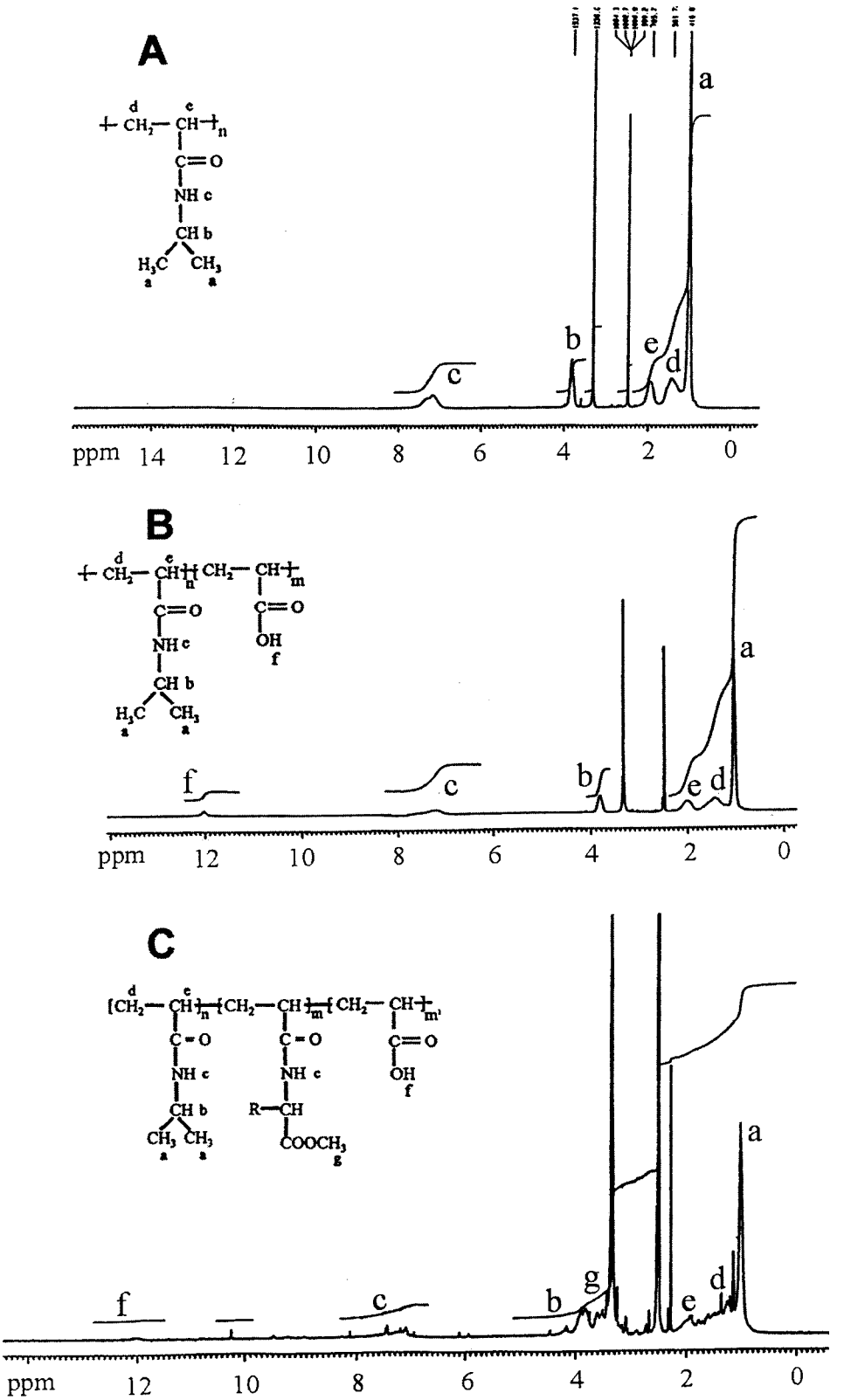


Figure 2 [1]H-NMR spectra of (A) PNIPAAm, (B) poly(NIPAAm-*co*-AAc), and (C) poly(NIPAAm-*co*-AAc)/Ala-OMe.

and poly(NIPAAm-*co*-AAc)/Ala-OMe conjugate, respectively. Table II shows the characteristic peaks in these spectra.

The amide and carboxylic acid absorption peaks can be identified in the FTIR spectra, which are specific to the NIPAAm and AAc components, respectively. The

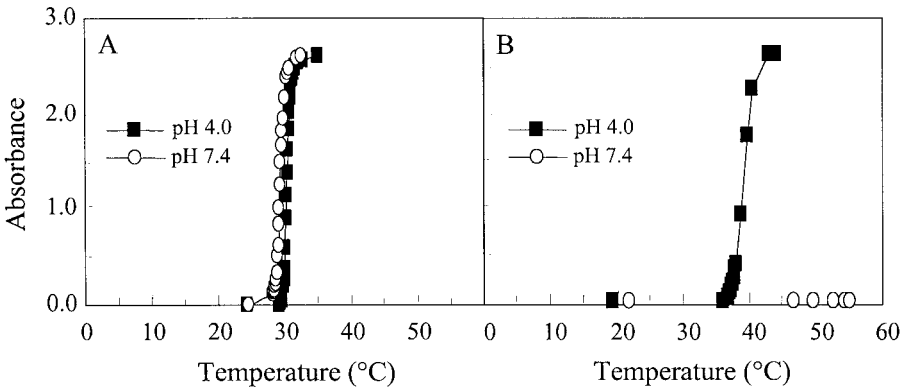


Figure 3 Temperature–absorbance curves at two different pHs: (A) PNIPAAm and (B) poly(NIPAAm-*co*-AAc).

amide peaks of the NIPAAm units in the homopolymer appeared at 1653 (amide I), 1549 (amide II), and 3440 cm^{-1} (N—H stretching). In the spectra of poly(NIPAAm-*co*-AAc), in addition to the characteristic peaks of the NIPAAm component, the characteristic C═O, O—H, and C—O stretching bands of the carboxylic acid groups of the AAc component appeared at 1723, 3358, and 1260 cm^{-1}, respectively. These characteristic peaks disappeared in the FTIR spectrum of the conjugates [Fig. 1(C)], in which the peak of the carbonyl groups (at 1709 cm^{-1}) and the C—O—C stretching (at 1215 cm^{-1}) of the amino acid/methyl ester can be observed. Note that similar peaks were observed in the FTIR spectra of other conjugates.

Figure 2(A–C) gives the ^{1}H-NMR spectra of the PNIPAAm homopolymer, poly(NIPAAm-*co*-AAc) copolymer, and poly(NIPAAm-*co*-AAc)/Ala-OMe conjugate, respectively. The proton chemical shifts of specific groups in the polymers and conjugate are also indicated in these spectra. Basically, the copolymer spectrum is superposed on the spectrum of the PNIPAAm homopolymer. In addition to the peaks observed in the homopolymer spectrum, a carboxylic acid proton peak appeared at δ = 12 ppm in the copolymer spectrum. The intensity of this peak was reduced in the spectrum of the conjugate, whereas the intensity of the peak at 7.2 ppm increased, representing an increase in the amide groups. Another indication for the conjugation was the appearance of a methyl ester signal around 3.7 ppm.

Stimulus-responsive behavior of the copolymer and conjugates

Figure 3 shows the absorbance changes of the homopolymer and copolymer solutions with the temperature. Note that each experiment was conducted at two different pHs (4.0 and 7.4).

Figure 3(A) shows that there was no significant effect of the variation of pH on the solubility of the homopolymer. The copolymerization of the temperature-responsive NIPAAm with the pH-responsive AAc resulted in copolymers sensitive to either pH or temperature [Fig. 3(B)]. The phase separation of poly(NIPAAm-*co*-AAc) shifted to higher temperatures as the pH of the solution increased.[14–16] An increase in the pH of the copolymer solution caused the ionization of carboxylic acid groups of AAc units in the copolymer chain. The ionized form of carboxyl groups (—COO^-) made the copolymer chains more hydrophilic, and this resulted in a rising LCST of the copolymer to higher temperatures (see Table III).

Figure 4(A,B) represents the stimulus-responsive behavior of the copolymer and its alanine methyl ester conjugates at pH 4.0 and 7.4, respectively. The LCST values determined from these graphs are given in Table III.

With the Ala-OMe conjugate, an observable decrease occurred in the LCST of poly(NIPAAm-*co*-AAc) at pH 4.0. The LCST value of the poly(NIPAAm–AAc) copolymer (37.7°C) was significantly higher than that of the PNIPAAm homopolymer (29.9°C) because of hydrophilic carboxylic acid groups coming from the AAc comonomer. The condensation reaction via these carboxylic acid groups caused an expected drop in the LCST value of the conjugate. The effect of the conju-

TABLE III
LCST Values of PNIPAAm and Poly(NIPAAm-*co*-AAc) and its Ala-OMe, Ala– Ala-OMe, and Ala–Ala–Ala-OMe Conjugates

Polymer/conjugates	LCST at pH 4.0[a] (°C)	LCST at pH 7.4[a] (°C)
PNIPAAm	29.9	28.7
Poly(NIPAAm-*co*-AAc)	37.7 ± 0.5	> 60
Ala-OMe Conjugate	35.1 ± 0.4	41.6 ± 0.3
Ala–Ala-OMe Conjugate	43.3 ± 0.1	46.2 ± 0.3
Ala–Ala–Ala-OMe Conjugate	45.8 ± 0.3	> 60

[a] Average of three repeated experiments plus or minus the standard deviation.

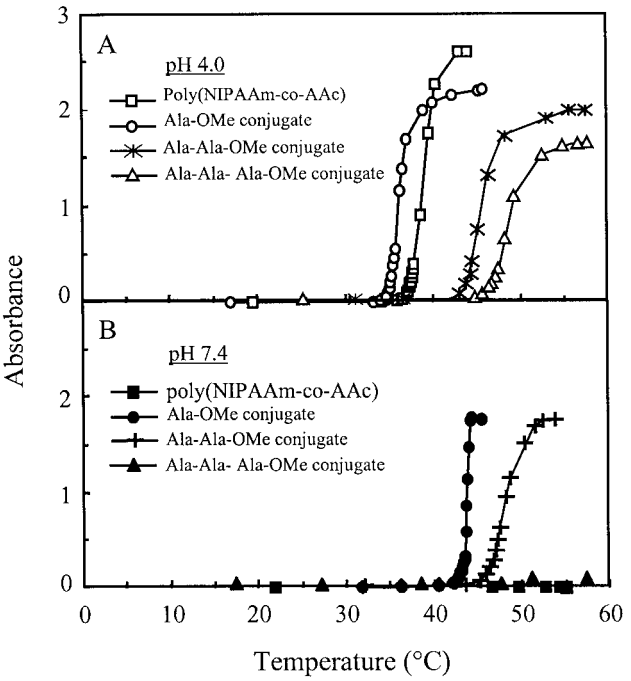


Figure 4 Temperature–absorbance curves of poly(NIPAAm-*co*-AAc) and its monopeptide, dipeptide, and tripeptide conjugates with alanine at two different pHs: (A) 4.0 and (B) 7.4.

gation reaction was quite profound at pH 7.4. A significant decrease in the LSCT value of the copolymer/Ala-OMe conjugate was observed with respect to the LCST of the unconjugated copolymer, and this was due to the loss of carboxylic acid groups of the copolymer in the reaction with Ala-OMe. This pH-dependent thermoresponsive behavior of the copolymer/Ala-OMe conjugate may be explained as follows. When the first amino acid was connected to the polymeric chain through the carboxylic acids, the number of carboxylic acid groups available decreased. This led to the decrease in the LCST at both pH 4.0 and 7.4 (compared with the LCST of the unconjugated copolymer) because the hydrophilicity of the copolymer chain coming from the carboxylic acid groups (both the protonated and ionized forms at pH 4.0 and 7.4, respectively) decreased. The side group of alanine (—CH_3) might also have had an effect on the LCST behavior of the monopeptide conjugates along with the number of carboxylic acids available.

In the case of the conjugates of the copolymer and the dipeptide and tripeptide, as the length of the oligopeptide chain increased, the LCST values of the conjugates at pH 4.0 and 7.4 increased significantly. This might have been due to the lower connection yields obtained with these conjugates (69 and 57% at the second and third steps, respectively). The yield of amino acid conjugation determined the number of carboxylic acid groups and the number of amide bonds after the conjugation reaction. As the connection yields decreased, the number of carboxylic acid groups increased, which led to the increase in the LCST at both pH 4.0 and 7.4. After each step of amino acid connection, the number of amide bonds formed on the conjugate increased. This increase was less when the connection yield was low. The increase in the number of amide bonds on the conjugate was expected to result in an increase in the LCST values at pH 4.0 and 7.4 because of the hydrophilic character of the amide bonds. As a result of the balance of these effects, the LCST values of the dipeptide and tripeptide conjugates shifted to the higher temperatures at pH 4.0 and 7.4.

CONCLUSIONS

In this study, we first synthesized a random copolymer of NIPAAm and AAc containing about 3.1 mmol of carboxylic acid groups per gram of the copolymer and having an number-average molecular weight of 1400 by a free-radical polymerization with AIBN as an initiator in DMF. Then, monopeptides, dipeptides, and tripeptides of alanine were connected to poly(NIPAAm-*co*-AAc) by the condensation of the amine group of methyl ester protected alanine to the carboxylic acid groups of the copolymer or the pendant deprotected alanine in the presence of a water-soluble carbodiimide. The reaction percentages of Ala-OMe with carboxylic acid groups were 93, 69, and 57% in the synthesis of the monopeptide, dipeptide, and tripeptide conjugates, respectively. FTIR and ^{1}H-NMR spectra revealed the formation of the conjugates. The connection of Ala-OMe caused a significant decrease in the LCST of the copolymer, especially at pH 7.4. The LCST of the Ala–Ala-OMe and Ala–Ala–Ala-OMe conjugates at pH 4.0 and 7.4 shifted to significantly higher temperatures.

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